

EXHIBIT 7

getically stable stem-loop structures resistant to processive 3' exonucleases (13). Biochemical analysis of USS stem-loops is needed to determine their potential role in mRNA stabilization or termination.

USSs are in genetic equilibrium with mutated sites containing one or more mismatches. In the absence of selection, the frequencies of mutated and nonmutated sites are simply those found in a random sequence. Any observed excess of sites must be accounted for by some form of selection. In *H. influenzae*, selection for donor DNA containing USS occurs at the cell surface. Thus, one restoring force for correct sites is transformation itself (14). If a cell in the population loses a site to mutation, that site will tend to be replaced with a correct site by transformation, because donor DNA carrying the correct site is preferentially taken up compared to donor DNA carrying the incorrect version of the site. An additional selective advantage might derive from the participation of a significant fraction of the sites in stem-loop structures with possible roles in transcription termination or regulation. A third selective advantage might come from any role that the USSs might play as recombinational hotspots, similar to the χ sites of *E. coli* (15).

The χ sites have the sequence 5'-GCT-GGTGG (plus orientation) in *E. coli* and are recognized by *recBCD* exonuclease V (15). Exonuclease V moves processively along the DNA, unwinding and cleaving the DNA until a χ site is encountered in the minus orientation. The enzyme then cleaves near the χ site and undergoes a structural change such that further cleavage is suppressed and the strands are unwound, producing a free 3' single strand that can synapse with homologous DNA to initiate recombination with the help of the RecA protein (15). In *E. coli*, the sites are distributed with a strong strand bias such that moving clockwise from the origin of replication, the sites are mostly in the plus orientation and counterclockwise they are mostly in the minus orientation (15). The average spacing of χ sites in *E. coli* is 5 kb (15). *H. influenzae* has genes homologous to the *recB*, *recC*, and *recD* genes of *E. coli* (1), and the *H. influenzae* exonuclease V has been purified and extensively studied (16, 17). Its properties are similar to those of the *E. coli* enzyme. USSs are frequent but lack the regional strand bias characteristic of the *E. coli* χ sites. The plus and minus sites appear to be randomly mixed (Fig. 1). Runs of plus USS sites or of minus USS sites do not exceed eight repeats in length, and the distribution of run lengths is about as expected by chance. On the other hand, a search of the *H. influenzae* genome reveals 98 copies of the sequence 5'-GCT-GTGG, 44 in the plus orientation and 54 in

the minus orientation, and only eight would be expected in each orientation by chance. However, only a weak strand bias of these putative plus and minus χ sites is seen relative to the origin. There are eight plus putative χ sites and 22 minus putative χ sites in 600 kb to the left of the *ori* (origin of replication) site, located at position 602 kb on the genome sequence (1), and 18 plus versus 21 minus putative χ sites in 600 kb to the right of *ori*. Whether *H. influenzae* and *E. coli* share the same χ site specificity will have to be determined by complementation of *recBCD* mutants of *E. coli* with *H. influenzae* genes.

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11. The nucleotide frequency of *H. influenzae* Rd is A = 0.308, T = 0.310, G = 0.192, and C = 0.190. The expected random frequency of the site AAGTGCGGT per genome is approximately $(0.31)^4 \times (0.19)^5 \times 1830121 = 4.2$. The expected frequency for both orientations of the site is then 8.4 per genome.
12. The expected random occurrence of a singly mutated USS is $[8 \times (0.19)^6(0.31)^3 + 9 \times (0.19)^5(0.31)^4 + 10 \times (0.19)^4(0.31)^5] \times 1.830,121 \times 2 = 254$.
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19. H.O.S. is an American Cancer Society Research Professor. This work was supported in part by research grants from the American Cancer Society and NIH. B.A.D. is supported by an NIH Training Grant.

16 May 1995; accepted 28 June 1995

Downloaded from www.sciencemag.org on January 24, 2007

Effects of the *obese* Gene Product on Body Weight Regulation in *ob/ob* Mice

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C57BL/6J mice with a mutation in the *obese* (*ob*) gene are obese, diabetic, and exhibit reduced activity, metabolism, and body temperature. Daily intraperitoneal injection of these mice with recombinant OB protein lowered their body weight, percent body fat, food intake, and serum concentrations of glucose and insulin. In addition, metabolic rate, body temperature, and activity levels were increased by this treatment. None of these parameters was altered beyond the level observed in lean controls, suggesting that the OB protein normalized the metabolic status of the *ob/ob* mice. Lean animals injected with OB protein maintained a smaller weight loss throughout the 28-day study and showed no changes in any of the metabolic parameters. These data suggest that the OB protein regulates body weight and fat deposition through effects on metabolism and appetite.

Mutation of the *obese* gene in the C57BL/6J mouse results in a syndrome that includes obesity, increased body fat deposition, hyperglycemia, hyperinsulinemia, and hypothermia (1). Parabiosis studies have suggested that the mutant obese mouse (*ob/ob*) lacks a blood-borne factor that could regulate adiposity by modulation of appetite and metabolism (2). Here we test the hypothesis

that the recently cloned *obese* gene (3) is involved in the regulation of adiposity by administering the OB protein to *ob/ob* mice.

The OB protein was expressed in *Escherichia coli* and purified to homogeneity as a 16-kilodalton monomer (4). The protein was dissolved in phosphate-buffered saline (PBS) (pH 7.4) and administered by daily intraperitoneal injection (0.1, 1.0, or 10.0 mg/kg) to 5-week-old C57BL/6J mice that were either homozygous (*ob/ob*) or heterozygous (+/?) for the *obese* gene mutation. The OB protein was also administered to 8-week-old, weight-stabilized normal C57BL/6J mice. Controls received equivolume (10 ml/kg) injections of PBS. Body weight, food

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intake, and water intake were measured every 24 hours during a 6-day preinjection baseline period, and throughout the 28-day injection period (5).

Injection of the OB protein resulted in a dose- and time-dependent reduction in body weight for all groups of mice (Fig. 1). A mixed design, repeated measures analysis of variance (ANOVA) revealed a significant overall effect of dose [$F(3,102) = 17.92; P < 0.0001$] and group [$F(2,102) = 13.64; P < 0.0001$], along with a significant (dose \times group \times time) interaction

[$F(84,1428) = 11.12; P < 0.0001$]. Body weight increased at different rates in the three groups of PBS-treated mice, with the *ob/ob* mice gaining weight most rapidly ($P < 0.003$ to < 0.04). Furthermore, all groups of mice showed a dose-dependent decrease in body weight gain over time with injection of the OB protein ($P < 0.0001$). The *ob/ob* mice that received the highest dose (10 mg/kg per day) decreased their body weight by 22.2% from preinjection baseline values, whereas those receiving PBS or the lowest dose of OB (0.1 mg/kg per day) maintained a 7.2% and 17.13%, respectively.

The reason for the apparent weight gain in the latter group is not clear. It should be emphasized, however, that there was a trend for other parameters normally associated with obesity (serum glucose and insulin levels, adiposity, and food intake) to be decreased in the *ob/ob* mice receiving the lowest dose of OB (0.1 mg/kg per day) relative to PBS controls.

The rate of weight gain was also significantly altered by the OB protein in the age-matched $+/?$ mice, as well as in the older $+/+$ mice. The $+/?$ mice receiving the highest dose of OB (10 mg/kg per day) showed a loss of 3.3% from their baseline weight, whereas the PBS controls showed a 7.7% increase from their baseline weight ($P < 0.001$). Although injection of the OB protein did not significantly decrease weight gain in $+/+$ mice, there was a non-significant trend toward weight loss: $+/+$ mice injected with the highest dose (10 mg/kg per day) maintained a loss of 5.3% from their baseline weight, and those in-

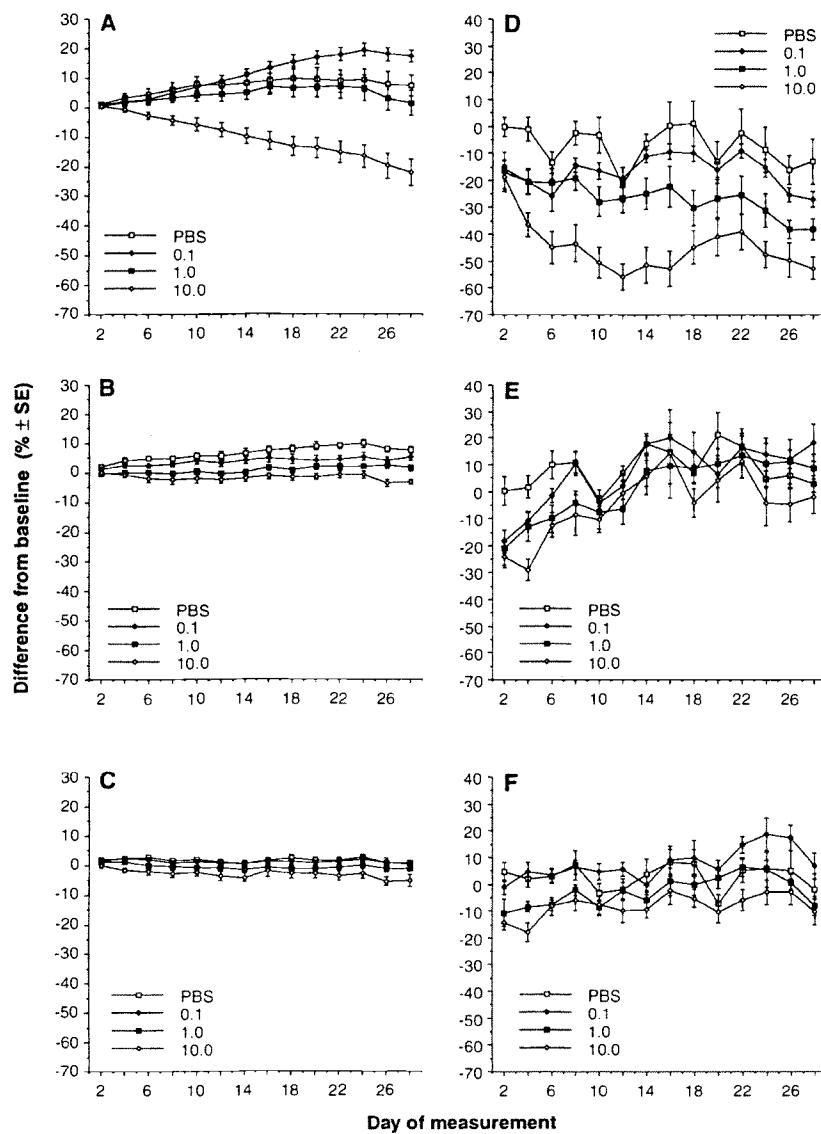


Fig. 1. Effects of OB protein administration on body weight in (A) C57BL/6J *ob/ob* mice, (B) C57BL/6J $+/?$ mice, and (C) C57BL/6J $+/+$ mice. Numbers indicate doses (mg/kg per day). Body weight is expressed as the percent difference from the body weight on day 6 of baseline measurement. The first injection was given on day 1. Baseline weights for each group of PBS-treated animals were: *ob/ob*, 27.95 ± 1.34 g; $+/?$, 18.8 ± 0.23 g; and $+/+$, 21.7 ± 0.25 g. Shown in (D), (E), and (F) is food intake as a percentage of baseline consumption for the three groups of mice. The average baseline food intake values per day for PBS-treated mice were: *ob/ob*, 4.23 ± 0.16 g; $+/?$, 2.97 ± 0.075 g; and $+/+$, 4.0 ± 0.12 g. The baseline body weights and food intake of other dose groups were not statistically different from those of the PBS groups. Each test group contained 8 to 10 mice.

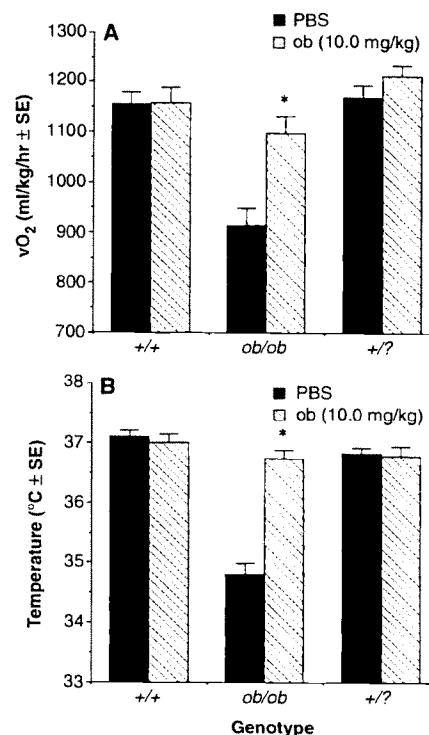


Fig. 2. Effects of OB protein administration on (A) vO_2 consumption and (B) body temperature in $+/+$, *ob/ob*, and $+/?$ mice. Body temperature was measured with a rectal thermistor. Oxygen consumption corresponds to the average volume of O_2 (vO_2) consumed during 15 1-min sampling periods. Measurements were taken in an airtight Oxymax chamber ($30.6 \times 10.2 \times 15.2$ cm) with an O_2 flow rate of 0.75 L/min (Columbus Instruments, Columbus, Ohio). Statistical differences were assessed by two-way ANOVA.

jected with PBS showed a 0.6% gain from their baseline weight.

These results suggest that *ob/ob* mice are more sensitive to the OB protein than are lean controls. The pharmacokinetic characteristics of the OB protein are currently unknown, so it remains possible, that its activity in lean mice could be increased by a different method of administration. Interestingly, constant infusion of the OB protein at a low dose (0.3 mg/kg/day) into 5-week-old *+/+* mice resulted in a maximal weight loss of 4.6% by day 6 of infusion ($P < 0.0001$). These mice maintained a small but significant weight loss ($2.2\% \pm 1.3$; $P < 0.0001$) throughout the 21 days of infusion (6).

The weight loss observed after injection of the OB protein was attributable in part to a reduction in food intake. A mixed-design ANOVA revealed that the OB protein significantly decreased food intake in a dose-dependent manner [$F(3,104) = 18.43$; $P < 0.0001$] (Fig. 1, D to F). This effect was more pronounced in *ob/ob* mice than in either group of lean mice ($P < 0.0001$).

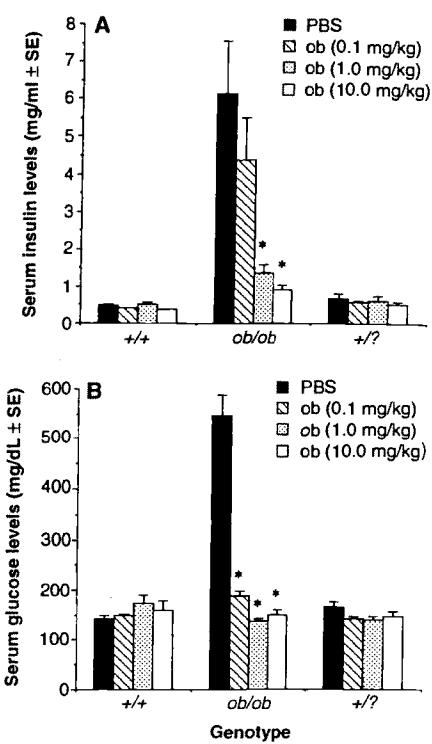


Fig. 3. Effects of OB protein administration on serum levels of (A) insulin and (B) glucose in *+/+*, *ob/ob*, and *+/?* mice. Insulin was measured by a radioimmunoassay system (Linco Research Laboratories, St. Charles, Missouri). Glucose was measured with a Hitachi 717 blood chemistry analyzer, using the hexokinase method (Boehringer Mannheim Biochemicals, Indianapolis, Indiana). Serum was taken by retro-orbital bleeds from fed mice. Statistical differences were assessed by two-way ANOVA.

Food intake for *ob/ob* mice injected with the highest dose of the OB protein (10 mg/kg per day) had dropped 52.6% below preinjection levels by day 28 of injection. Although *+/?* mice showed a decrease in food intake during the first 4 days of treatment, their food consumption was not different from either their own baseline levels or from PBS-treated controls by day 12 of treatment. A similar pattern was observed with the *+/+* mice.

Water intake was also significantly decreased by administration of the OB protein, but only in the *ob/ob* mice. This effect was due primarily to the fact that OB protein-treated *ob/ob* mice did not increase water intake over time, as did their diabetic PBS-treated *ob/ob* counterparts ($P < 0.008$ to < 0.0001). The water intake of *+/?* or *+/+* mice was not affected by the OB protein. Thus, the effect of the protein on water intake was different from its effects on food intake and body weight, both of which decreased in a dose-dependent manner over time in all groups of mice.

To test whether the OB protein altered metabolic or endocrinological parameters in addition to appetite, we measured O_2 consumption, body temperature, total locomotor activity, and serum insulin and glucose levels during the third week of OB protein administration. The PBS-treated *ob/ob* mice showed significantly lower O_2 consumption than lean counterparts ($P < 0.001$), but this parameter was normalized in *ob/ob* mice receiving the highest dose of OB protein (10 mg/kg per day) (Fig. 2A). In contrast, the OB protein did not affect O_2 consumption in either group of lean mice.

PBS-treated *ob/ob* mice were significantly hypothermic in comparison to lean mice

($P < 0.0001$). However, treatment with even the lowest dose of the OB protein raised the body temperature of *ob/ob* mice to that of lean mice (Fig. 2B). The OB protein did not affect body temperature in either group of lean mice.

Locomotor activity was assessed in these mice (7) during the same week that O_2 consumption and body temperature were monitored. PBS-treated (*ob/ob*) mice were significantly hypoactive ($P < 0.0001$) in comparison to lean *+/+* or *+/?* mice, but the group of *ob/ob* mice receiving the highest dose of OB protein (10 mg/kg per day) increased their total activity to the level observed in their lean counterparts ($P < 0.0001$). The OB protein did not affect total activity in lean mice and did not induce any form of stereotypic behavior. These activity data, along with the observation that metabolic indices were normalized in *ob/ob* mice and were unaltered in lean mice, argue against generalized toxicity as a mechanism for weight loss by the OB protein.

Serum insulin and glucose levels were also decreased in a dose-dependent manner by the OB protein in the *ob/ob* mice, suggesting that pancreatic function was normalized in these usually hyperinsulinemic and hyperglycemic animals (Fig. 3, A and B). Indeed, *ob/ob* mice had significantly higher serum insulin and glucose levels than *+/?* or *+/+* mice ($P < 0.0001$). Administration of even the lowest dose of OB protein reduced glucose levels in *ob/ob* mice by 66% and insulin levels by 41%. At the highest dose (10 mg/kg per day), the OB protein normalized insulin and glucose levels to those seen in *+/+* mice ($P < 0.0001$). Neither insulin or glucose levels were significantly altered in lean mice.

Table 1. Effects of OB protein administration on carcass composition in C57BL/6J *+/+*, *ob/ob*, and *+/?* mice. Carcass components are presented as percentages of total body weight and as absolute weights.

Dose of OB (mg/kg per day)	Water (%)		Fat (%)		Lean mass (%)		Water (g)	Fat (g)	Lean mass (g)
	<i>+/+</i>	<i>ob/ob</i>	<i>+/+</i>	<i>ob/ob</i>	<i>+/+</i>	<i>ob/ob</i>			
0 (PBS)	56.30 ± .49		17.86 ± 1.2		25.80 ± .70		12.54 ± .23	3.96 ± .27	5.74 ± .19
0.1	52.35 ± .50*		21.27 ± .72*		26.38 ± .70		11.51 ± .16	4.67 ± .16	5.8 ± .17
1	54.29 ± .87		18.40 ± .77		27.29 ± .60		11.89 ± .34	4.01 ± .16	5.96 ± .09
10	52.53 ± .84*		19.33 ± 1.3		28.10 ± .80*		10.83 ± .33*	3.96 ± .25	5.79 ± .21
(+/-)									
0 (PBS)	56.0 ± .78		28.17 ± .86		15.80 ± .40		16.81 ± .67	8.42 ± .34	4.74 ± .23
0.1	52.5 ± .42*		25.91 ± 1.1		14.70 ± .50		19.72 ± .44	8.58 ± .38	4.86 ± .18
1	57.0 ± 2.0		24.73 ± 1.7*		18.23 ± .70*		15.52 ± .68	6.72 ± .49*	4.93 ± .17
10	54.17 ± .92		22.70 ± .93*		28.10 ± .80*		11.18 ± .54	4.7 ± .31*	4.68 ± .13
(+/?)									
0 (PBS)	52.25 ± .73		22.56 ± .76		25.20 ± .70		10.84 ± .18	4.67 ± .15	5.24 ± .18
0.1	59.44 ± .86*		21.92 ± .50		25.60 ± .50		10.51 ± .22	4.39 ± .17	5.11 ± .10
1	52.42 ± .98		20.49 ± .77		27.10 ± .40		9.70 ± .24	3.78 ± .13*	5.10 ± .11
10	55.09 ± 1.4		17.88 ± 1.4*		27.0 ± .70		9.97 ± .28	3.24 ± .28*	4.89 ± .14

* $P < 0.03$ to < 0.0002 .

If the OB protein is a "sensor" of adiposity (3, 8), exogenous OB protein should reduce adiposity in *ob/ob* mice. The percent body fat (9) was normalized in *ob/ob* mice injected with OB protein in a dose-dependent fashion as assessed by two-way ANOVA (Table 1). The percent body fat was significantly decreased both in *ob/ob* mice (treated with OB protein at 10 mg/kg per day or 1 mg/kg per day; $P < 0.0001$) and in *+/?* mice (treated with OB protein at 10 mg/kg per day; $P < 0.002$). The percent body fat of *+/+* mice was not significantly changed by the OB protein at any dose.

The *ob/ob* mice injected with the OB protein also showed a dose-dependent increase in lean mass as a percent of body weight ($P < 0.017$ to < 0.0001). There was also a significant increase in percent lean mass in *+/+* mice injected with the highest dose of OB protein (10 mg/kg per day) ($P < 0.026$), and a nonsignificant trend toward the same effect in the *+/?* mice. There were no significant changes in absolute lean mass for any group, however. Water as a percentage of carcass weight was not affected in a consistent dose-dependent manner by the OB protein in any group of mice.

Our data support the hypothesis that the OB protein plays a pivotal role in the regulation of body weight and adiposity in mice. Its mechanism of action is likely to be more complex than appetite suppression, since (i) lean *+/+* and *+/?* mice maintained a lower body weight even though their food intake had recovered to baseline values early in the course of the study, and (ii) the lowest dose of OB protein normalized both body temperature and serum glucose levels in *ob/ob* mice even though weight and food intake were not significantly reduced. The latter observation may indicate that the metabolic and hormonal effects of the OB protein precede its effects on appetite and body weight. Further understanding of the protein's mechanism of action will require a more direct examination of its effects on hypothalamic-pituitary function and identification of its receptor.

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- Recombinant murine OB protein was expressed in *Escherichia coli* in inclusion bodies. The protein was allowed to fold in solubilized inclusion bodies [H. Lu, C. Clogston, L. Merewether, L. Narhi, T. Boone, in *Protein Folding: In Vivo and In Vitro*, J. Cleland, Ed. (American Chemical Society, Washington, DC, 1993), vol. 526, chap. 15] and was purified by ion exchange and hydrophobic interaction chromatography to 95% purity as assessed by SDS-polyacrylamide gel electrophoresis.
- Mice were given ground rodent chow (PMI Feeds, Inc., St. Louis, MO) in powdered food feeders (Allentown Caging and Equipment, Allentown, PA). Body weight and food and water intake were measured at 2:00 p.m. each day. Mice were single-housed and were maintained under conditions that are in accordance with the guidelines set for animal care by Amgen's Institutional Animal Care and Use Committee.
- Five-week-old *+/+* mice were implanted subcutaneously with osmotic minipumps (1007D; Alzet, Palo Alto, CA). These pumps delivered OB protein (0.3 mg/kg per day) or PBS for 7 days at a rate of 0.5 µl/hour. Pumps were replaced every 7 days. Body weight was monitored every 2 days. There were five mice in each group.
- Locomotor activity was measured as in [D. Britton et al., *Pharmacol. Biochem. Behav.* **34**, 779 (1989)]. Mice were placed in individual open cages that contained an inverted test tube rack and observed every 30 s for a 15-min period. Mice were scored for a battery of activities, including climbing, grooming, sniffing, rearing, and walking. Grooming and sniffing were defined as stereotypic activities. Total activity was defined as the summary of all activities in the battery. We made 26.2 ± 0.52 and 22.6 ± 1.19 observations of total activity for PBS-treated *+/+* and *+/?* mice, respectively. Administration of even the highest dose of OB protein did not substantially alter activity levels in lean mice. In contrast, we made only 8.9 ± 1.92 observations of total activity for PBS-treated *ob/ob* mice. Two-way ANOVA showed that *ob/ob* mice treated with OB protein (10 mg/kg per day), however, were almost as active as lean mice (20.3 ± 2.42 observations). There were no dose-related differences in the number of stereotypic activities observed for any group.
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- Carcass analysis was conducted as in A. I. Leshner, V. A. Litwin, and R. L. Squibb [*Brain Res.* **9**, 281 (1972)]. Water composition was determined by subtraction of carcass weight before and after a 4-day dehydration period. Fat was extracted with ethyl ether and ethyl alcohol from a preweighed portion of the ground, dried carcass, so that percent fat could be calculated from the amount of material remaining after the extraction procedure. Lean mass was defined as the proportion of ground carcass that remained after dehydration and ether extraction.
- We thank L. Ross for expert technical assistance.

26 May 1995; accepted 29 June 1995

Weight-Reducing Effects of the Plasma Protein Encoded by the *obese* Gene

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The gene product of the *ob* locus is important in the regulation of body weight. The *ob* product was shown to be present as a 16-kilodalton protein in mouse and human plasma but was undetectable in plasma from C57BL/6J *ob/ob* mice. Plasma levels of this protein were increased in *diabetic* (*db*) mice, a mutant thought to be resistant to the effects of *ob*. Daily intraperitoneal injections of either mouse or human recombinant OB protein reduced the body weight of *ob/ob* mice by 30 percent after 2 weeks of treatment with no apparent toxicity but had no effect on *db/db* mice. The protein reduced food intake and increased energy expenditure in *ob/ob* mice. Injections of wild-type mice twice daily with the mouse protein resulted in a sustained 12 percent weight loss, decreased food intake, and a reduction of body fat from 12.2 to 0.7 percent. These data suggest that the OB protein serves an endocrine function to regulate body fat stores.

Higher vertebrates maintain a constant adipose tissue mass with precision (1, 2). The characteristics of the cloned mouse *obese* gene (*ob*) suggest a molecular mech-

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anism for this regulation (3). Defects in the *ob* gene lead to a marked increase in adipose tissue mass as part of a syndrome that resembles morbid obesity in humans (4). We now show that the OB protein is present in plasma and that daily injections of the recombinant protein reduce body weight and adipose stores in *ob/ob* and wild-type mice.

The OB protein from normal mouse plasma is present primarily as a monomer with a molecular size of ~16 kD, but was not detected in plasma from C57BL/6J *ob/ob* mice that have a nonsense mutation at codon 105 (Fig. 1A) (5–7). An increase in the level of circulating protein was observed in *db/db* mice relative to lean control animals (Fig. 1A). The *db* mutation results in an obese phenotype identi-